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1. **Holland et al. Anal. Biochem. 1994, 222(2), pp. 516-518.**
2. **Peterson et al. Anal. Biochem. 1999 (7/1/99), 271(2), pp. 131-136.**
3. **Antonsson et al. Anal. Biochem. 1999 (2/15/99), 267(2), pp. 294-299.**
4. **Park et al. Anal. Biochem. 1999 (4/10/99), 269(1), pp. 94-104.**

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Maurie Garcia, Ph.D.
Patent Examiner, Art Unit 1627
Crystal Mall 1, Rm. 8A12 (703) 308-0065

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Homogeneous Proximity Tyrosine Kinase Assays: Scintillation Proximity Assay versus Homogeneous Time-Resolved Fluorescence

Young-Whan Park,[1] Richard T. Cummings, Lin Wu, Song Zheng, Patricia M. Cameron, Andrea Woods, Dennis M. Zaller, Alice I. Marcy, and Jeffrey D. Hermes

Department of Molecular Design and Diversity, Merck Research Laboratories, P.O. Box 2000, Rahway, New Jersey 07065-0900

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Two homogeneous proximity assays for tyrosine kinases, scintillation proximity assay (SPA) and homogeneous time-resolved fluorescence (HTRF), have been developed and compared. In both formats, the kinase assay was performed using biotinylated peptide substrate, ATP ([^{33}P]ATP in the case of SPA), and tyrosine kinase in a 96-well assay format. After the kinase reaction was stopped, streptavidin-coated SPA beads or europium cryptate-labeled anti-phosphotyrosine antibody and streptavidin-labeled allophycocyanin were added as detection reagents for SPA or HTRF assays, respectively. Since the assay signal was detected only when the energy donor (radioactivity for SPA, Eu for HTRF) and the energy acceptor molecules (SPA beads for SPA, allophycocyanin for HTRF) were in close proximity, both assays required no wash or liquid transfer steps. This homogeneous ("mix-and-measure") nature allows these assays to be much simpler, more robust, and easier to automate than traditional protein kinase assays, such as a filter binding assay or ELISA. Both assays have been miniaturized to a 384-well format to reduce the assay volume, thereby saving the valuable screening samples as well as assay reagents, and automated using automated pipeting stations to increase the assay throughput. Several advantages and disadvantages for each assay are described. © 1999 Academic Press

Modern high-throughput screening to support drug discovery requires ultrafast, yet highly robust assay methods. Homogeneous formats, requiring only simple additions of reagents (i.e., "mix-and-measure"), have become popular because they do not require time-consuming and error-prone wash steps. For example, three distinct homogeneous assay formats recently reported for tyrosine kinases include scintillation proximity assay (SPA)[2] (1–6), fluorescence polarization (FP) (7, 8), and a particular form of fluorescence resonance energy transfer (FRET) termed homogeneous time-resolved fluorescence (HTRF) (9, 10). Common to both FP and HTRF methods is the use of an anti-phosphotyrosine antibody to allow the product of the kinase reaction, a phosphorylated peptide, to be detected. A signal is observed only when the product peptide is bound to the antibody. In FP format, the phosphopeptide is recognized by the antibody and, because of the increase in mass, tumbles more slowly yielding a polarized fluorescence signal (11, 12). In the HTRF format the antibody is labeled with europium cryptate to function as the fluorescence energy donor with peptide conjugated to the allophycocyanin acceptor. By contrast, SPA uses a radioisotope as an energy donor and scintillant-coated SPA beads as an energy acceptor. In both SPA and HTRF formats, the energy transfer only occurs when the energy donor and energy acceptor molecules are in proximity (bound state).

Protein tyrosine kinases play a critical role in many cellular signal transduction pathways, and have re-

[1] To whom correspondence and reprint requests should be addressed. Fax: (732) 594-5042. E-mail: young_whan_park@merck.com.

[2] Abbreviations used: SPA, scintillation proximity assay; HTRF, homogeneous time-resolved fluorescence; FRET, fluorescence resonance energy transfer; FP, fluorescence polarization; Eu(K)-PY20, europium cryptate-labeled anti-phosphotyrosine antibody; streptavidin-XL665, streptavidin-labeled allophycocyanin; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ELISA, enzyme-linked immunosorbent assay; LCB, aminohexanoyl biotin or long-chain biotin.

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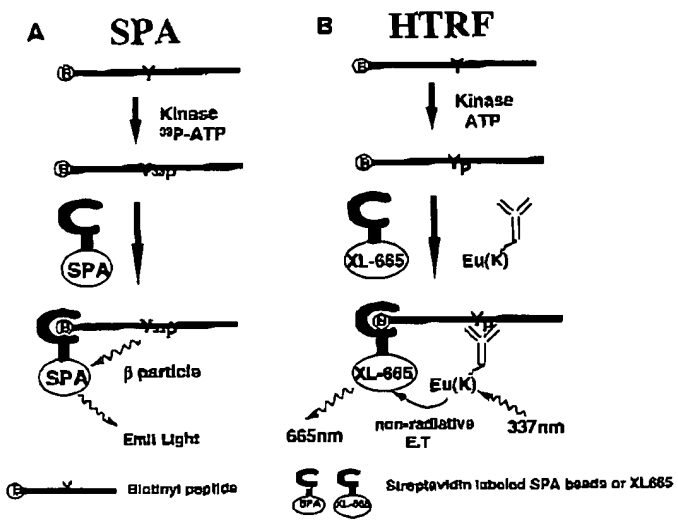

SCHEME 1. (A) SPA tyrosine kinase assay format, (B) HTRF tyrosine kinase assay format.

cently become popular for drug discovery programs. Hundreds of tyrosine kinases have been identified and the numbers may soon be greatly increased by genomic research (13, 14). To find an inhibitor for a specific tyrosine kinase via random screening, a large number of assay points will be required. Therefore a simple and fast assay format, such as a homogeneous assay, will be the best choice for the tyrosine kinase assay.

In this article, the SPA and the HTRF techniques which have been applied to develop homogeneous tyrosine kinase assays are compared. General assay formats are illustrated in Scheme 1. The SPA tyrosine kinase assay used ^{33}P as an energy donor and scintillant-coated SPA beads as an energy acceptor (Scheme 1A) (15). Biotinylated peptide substrate is phosphorylated by kinase with [^{33}P]ATP. The ^{33}P-labeled biotinylated peptide product is captured by streptavidin-coated SPA beads, bringing the energy donor molecules in close proximity to the scintillant-coated beads resulting in the emission of light. Conceptually the HTRF tyrosine kinase assay format is very similar to SPA (Scheme 1B). While SPA makes use of ^{33}P and streptavidin-labeled SPA beads, HTRF utilizes europium cryptate-labeled anti-phosphotyrosine antibody [Eu(K)-PY20] and streptavidin-labeled allophycocyanin (streptavidin-XL665) as a fluorescence donor and fluorescence acceptor, respectively (15). After the kinase catalyzes phosphorylation of the biotinylated peptide substrate, the Eu(K)-PY20 and streptavidin-XL665 are brought into close proximity by virtue of the interaction of each with the phosphorylated product peptide. A nonradioactive fluorescence energy transfer occurs between Eu(K) and XL665 when the Eu(K) is excited at 337 nm. A long-lived, 665-nm energy transfer signal results and correlates with the amount of phosphorylated peptide product formed.

MATERIALS AND METHODS

[γ-^{33}P]ATP (Redivue adenosine 5′-[γ-^{33}P] triphosphate triethylammonium salt, ≈2000 Ci/mmol) and streptavidin-coated SPA beads (typical capacity 100–150 pmol biotin/mg) were purchased from Amersham. *N*-LCB-EQEDEPEGDYEEVLE-NH_2 (peptide A1, LCB = aminohexanoylbiotin), *N*-LCB-EQEDEPEGIYGVLF-NH_2 (peptide B), their analog peptides (Table 1), and corresponding phosphotyrosine peptides were synthesized using an Applied Biosystem's 433A peptide synthesizer using FastMOC chemistry. Streptavidin-labeled XL665 (a modified allophycocyanin) and europium cryptate-labeled anti-phosphotyrosine antibody [(Eu(K)-PY20] were purchased from CIS-Bio (France) or prepared in-house (16). Anti-phosphotyrosine antibody PY20 was purchased from Transduction Laboratories (Lexington, KY). Tartrazine, methyl red, methyl orange, Chicago sky blue 6B, and naphthol green B were purchased from Aldrich (Milwaukee, WI). Staurosporine was purchased from Calbiochem (San Diego, CA).

Protein expression and purification. cDNA encoding amino acids 235–501 (catalytic domain) of human Lck and amino acids 2–619 (full-length) of human ZAP-70 were obtained by RT–PCR of total RNA isolated from Jurkat T cells. For ZAP-70, nucleotide sequences encoding a FLAG epitope recognized by the M2 monoclonal antibody were incorporated into the forward PCR primer. For Lck, nucleotide sequences encoding an SV40 large T antigen epitope recognized by the KT3 monoclonal antibody were incorporated into the reverse primer. Each cDNA was subcloned downstream of the metallothionine promoter in the pS2neo vector. The recombinant cDNA vectors were introduced into *Drosophila* S2 cells by calcium phosphate precipitation. Stable transformants were selected with 1.5 mg/ml active G418. Single cell colonies were isolated by seeding cells onto soft agar. The transformants were grown to a density of at least 4 million cells per milliliter in spinner flasks and then induced overnight with 1 mM copper sulfate. Cells were harvested by centrifugation and lysed by douncing at 2–4 × 10^8 cells/ml in 50 mM Tris buffer, pH 7.6, containing 10% glycerol, 1 mM DTT, and a protease inhibitor cocktail (Complete, Boehringer Mannheim) for Lck and in the same buffer supplemented with 150 mM NaCl for ZAP-70. The lysate was centrifuged at 100,000*g* for 1 h at 4°C and the supernatant was collected and passed through a 5-μm filter. Lck was purified by applying 20 ml of S100 to a 4-ml anti-KT3 column which had been equilibrated in 50 mM Tris buffer, pH 7.6, containing 10% glycerol and 1 mM DTT.

TABLE 1

Sequences of the HS1 Peptide Analogs and Their Relative Activities for ZAP-70 Kinase Reaction in SPA and HTRF Assay Formats

Peptide name	Peptide sequence	Rel. activity[a] in gel[b]	in SPA	in HTRF
A1	EQEDEPEGDYEEVLE	100	100	100
A2	EQEDEPEGDYWEWLE	102	97	390
A3	EQEDEPEGDYPEWLE	19	19	62
A4	EQEDEPEGDYEEWLE	115	125	169
A5	EQEDEPEGDYFEWLE	75	88	703

[a] All the relative activities were normalized to the parent peptide, HS1 (A1).

[b] Kinase reaction was performed with exactly the same conditions as in the SPA kinase assay (see Materials and Methods for detailed SPA conditions). After incubation for 40 min at room temperature, the assay mixture was stopped by adding gel-loading buffer, loaded on a 10–20% Novex Tricine gel, and quantified with an InstantImager (Packard).

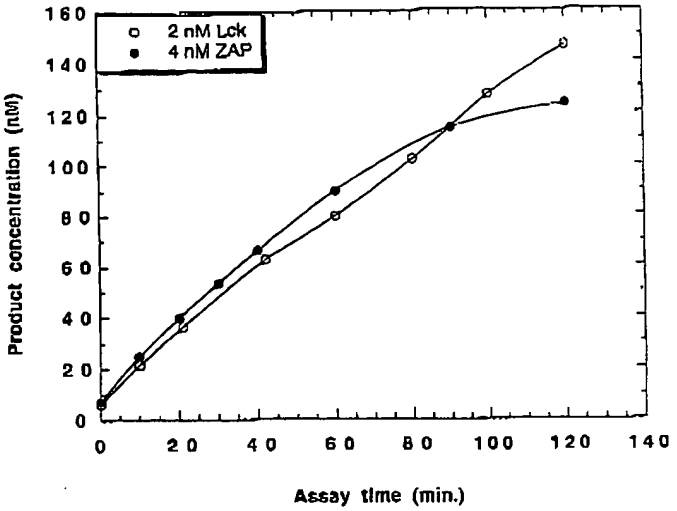

FIG. 2. Time course of Lck kinase assay reaction in SPA format. Kinase reaction using 2 μM ATP, 0.75 μM peptide substrate, 0.2 μCi [^{33}P]ATP, and 2 nM Lck (or 4 nM ZAP-70) in assay buffer was stopped at each time point by adding stop buffer containing SPA beads.

kinase reactions with concentration of ATP ranging from 0.5 to 64 μM at 2 μM peptide. Calculated $K_{m,app}$ values for ATP were 33.4 and 30.8 μM for ZAP-70 and lck, respectively. $K_{m,app}$'s for peptide were measured from the kinase reactions with concentration of peptide from 1 to 128 μM at 2 μM ATP, and were 9.0 and 16.8 μM for ZAP-70 and lck, respectively. Using this K_m information, assay conditions were set at sub-$K_{m,app}$ concentrations, 2 μM ATP and 0.75 μM peptide substrate, to provide the most sensitive screen for inhibitors which are competitive with ATP and/or peptide substrate. Linearity of the kinase activity is shown in Fig. 2. While lck enzyme activity was linear for up to 2 h at room temperature, ZAP-70 activity was linear only for 1 h followed by a progressive decrease in the reaction rate. Two nanomolar lck or 4 nM ZAP-70 resulted in a signal about 10-fold higher than back-

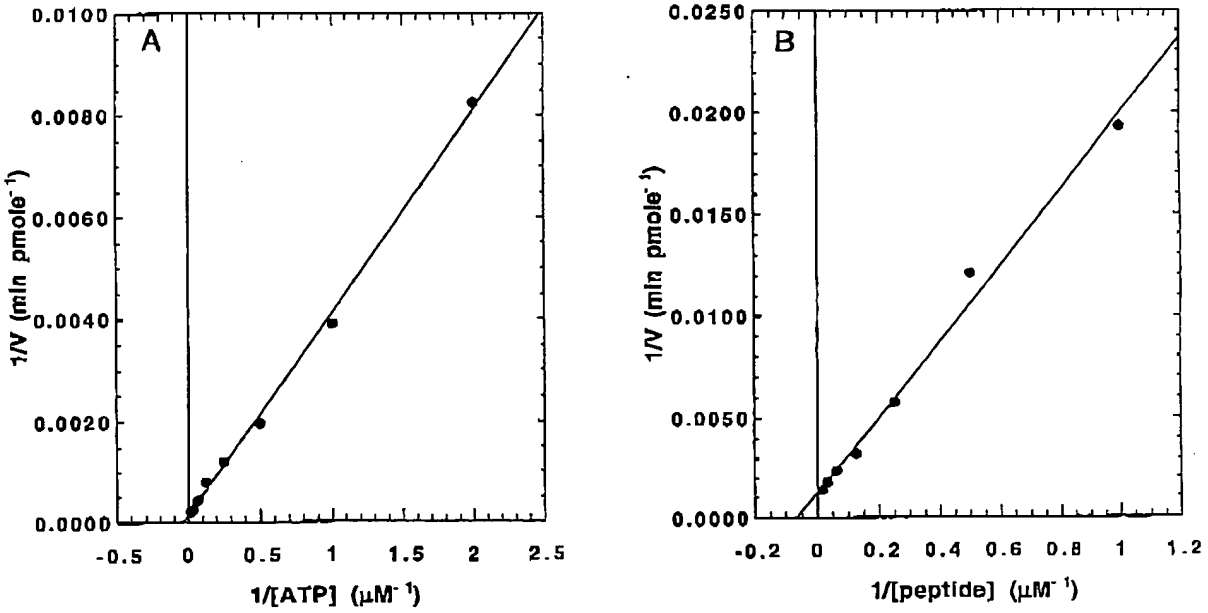

FIG. 1. Lineweaver–Burk analysis of SPA lck kinase assay using (A) 0.5, 1, 2, 4, 8, 16, 32, and 64 μM ATP concentrations at 2 μM peptide, and (B) 1, 2, 4, 8, 16, 32, 64, and 128 μM peptide concentrations at 2 μM ATP.

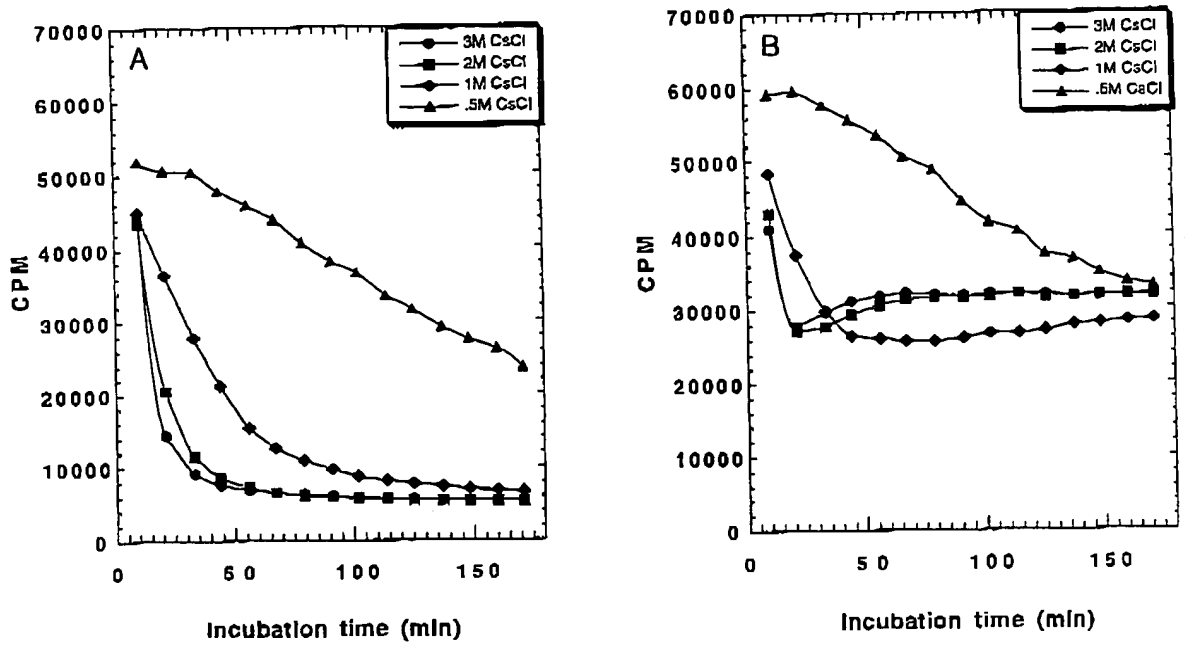

FIG. 3. Bead floatation method in SPA kinase assay. Kinase reactions were performed in a 100-μl reaction containing 2 μM ATP, 0.75 μM peptide B, 0.2 μCi [^{33}P]ATP, and 2 nM Lck. After an incubation for 40 min at room temperature, the kinase reaction mixture was stopped by adding a 50-μl stop buffer containing 50 mM Hepes, 50 mM EDTA, 0.1% BSA, 0.1% Triton X-100, pH 7.25, and SPA beads (Amersham, Cardiff, England, 20 mg/ml with 100–150 pmol/mg biotin binding capacity). To the stopped kinase reaction, 100 μl of CsCl solution was added in different concentrations. (A) Background signal (no enzyme). (B) Assay signal.

ground after a 40-min incubation at room temperature. This time point was in a linear portion of the reaction time course and is used for all kinase assays unless otherwise noted. All the other src family tyrosine kinases tested also showed linear activity for at least 1 h (data not shown). To capture all the biotinylated peptide substrate (both phosphorylated and nonphosphorylated), an amount of the streptavidin-coated SPA beads corresponding to an excess of biotin binding sites was used in all kinase reactions. Streptavidin is a tetramer, and each subunit can bind one biotin with a very high binding affinity ($K_a \approx 10^{15}$ M^{-1}). Although the high-affinity streptavidin capture step is unlikely to alter the assay result, we compared our SPA assay results with a more direct measurement using a gel electrophoresis kinase assay performed in parallel. The kinase assay was performed with the exact same conditions as in the SPA kinase assay, and the radioactive assay mixture was analyzed by gel electrophoresis to quantify the phosphorylated product. The relative activities of ZAP-70 with HS1 peptide analogs as substrates in the gel assay format were almost identical to the relative activities from the SPA format (Table 1). This comparison showed that the streptavidin capture step in SPA format did not affect the assay result.

SPA bead floatation method. Since the SPA kinase assay uses ^{33}P, a higher energy radioisotope than the typically used ^{3}H isotope, it was necessary to centrifuge the SPA beads down to the bottom of the assay plate ("settling") to pack the beads into a localized space and collect the proximity assay signal most efficiently. This packing of the SPA beads also serves to reduce the background signal due to the bulk of free [^{33}P]ATP in the assay solution (2–4). However, this method requires manually moving the assay plates to a centrifuge, and is not compatible with full automation. A bead floatation method has been developed to eliminate this inconvenience (5, 6). In the floatation method, CsCl was added to the assay solution after the kinase reaction was stopped with quench buffer containing SPA beads. The CsCl causes the beads to float to the top of the assay solution ("reverse-settling") by increasing the density of the solution. Figure 3 shows changes in the background as well as the assay signals over a 3-h incubation time period with 0.5, 1, 2, and 3 M CsCl. At 0.5 M CsCl concentration, the background and assay signals did not reach equilibration even after a 3-h incubation. However, at the higher CsCl concentrations, the signal stabilized in less than 1 h. Two M CsCl appears optimal (Fig. 3). Use of more than 2 M CsCl resulted in a similar stabilization time. This optimal concentration of CsCl is valid only for PVT-SPA beads and one should expect this to be different for other types of beads depending on their particular density. The stabilized assay signal, as well as the signal to

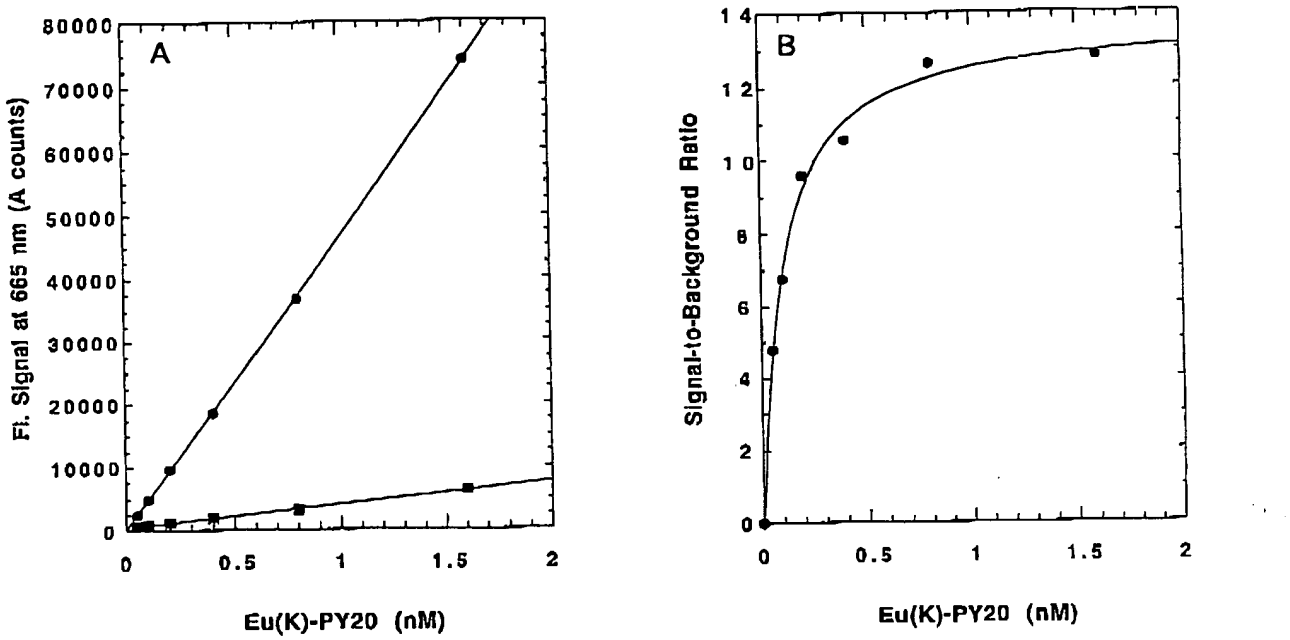

FIG. 4. Titration of Eu(K)-PY20 in HTRF kinase assay. 50 μl of kinase assay reactions containing 2 μM peptide B, 10 μM ATP, and 0.5 nM Lck was incubated for 40 min at room temperature. 150 μl of stop buffer solutions containing streptavidin-XL665 and different concentrations of Eu(K)-PY20 was then added to a final concentration of result 500 nM streptavidin-XL665 and 0.2–1.5 nM Eu(K)-PY20.

background ratio, were very close to the ones obtained from the centrifugation method.

HTRF tyrosine kinase assay. The HTRF kinase assay utilizes the labeled reagents streptavidin-XL665 and Eu(K)-PY20 as an energy acceptor and an energy donor, respectively. The optimal concentrations have been determined by titration of each of the labeled reagents in the kinase assay. *A* counts (fluorescence signal at 665 nm) linearly increased as the Eu(K)-PY20 concentration was increased (Fig. 4A). This result was expected since the estimated phosphorylated assay product at the given assay condition was much larger than the antibody concentrations used (HPLC integration of the kinase reaction mixture showed that ≈13% of the substrate was phosphorylated under the same assay conditions, data not shown). Although the maximal assay signal-to-background ratio was obtained at 0.8 nM (Fig. 4B), 0.4 nM was selected as a final Eu(K)-PY20 concentration to minimize reagent cost. This concentration of Eu(K)-PY20 still produced a signal-to-background ratio greater than 10:1. As in the SPA kinase assay, an excess of streptavidin-XL665 over total peptide concentration was used. Titration of streptavidin-XL665 was used to establish this saturating amount (data not shown). The lck kinase assay signal was monitored as a function of assay time and enzyme concentration (Fig. 5), and was linear under these conditions until the antibody was titrated with phosphorylated product peptide. Figure 5 shows the remarkable sensitivity of the HTRF assay. With 20 pM lck, greater than 10:1 signal-to-background ratio was obtained at 40-min incubation. To obtain a similar-signal-to-background ratio, the SPA kinase assay required 2 nM of lck (Fig. 2). This result demonstrates that the HTRF assay is two orders of magnitude more

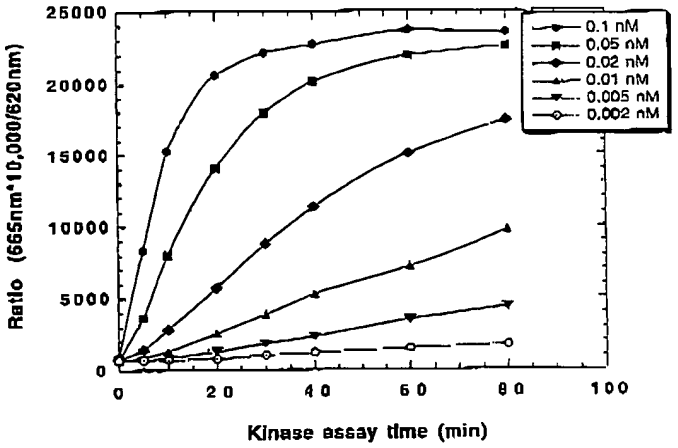

FIG. 5. Time course of Lck kinase reaction in HTRF format. 500-μl kinase reactions containing 2 μM peptide B, 10 μM ATP were started by adding Lck to a final concentration of 0, 0.002, 0.005, 0.01, 0.02, 0.05, 0.1, and 0.2 nM. 50-μl aliquots of the kinase reaction mixture were stopped by adding 150 μl of stop buffer containing 670 nM streptavidin-XL665 and 0.53 nM Eu(K)-PY20 after 0, 5, 10, 20, 30, 40, 60, and 80 min of incubation at room temperature. Ratio was calculated by (fluorescence at 665 nm × 10,000) / fluorescence at 620 nm.

sensitive than the SPA assay format under optimized lck kinase assay conditions. Although the difference in absolute sensitivity will be decreased to one order of magnitude with a correction for the difference between the two assay conditions (10 μM ATP and 2 μM of peptide were used for the HTRF while 2 μM of ATP and 0.75 μM of peptide were used for the SPA), the practical assay sensitivity will remain similar because of limits on the concentration of radioactivity and SPA beads. Even at 2 pM of lck, the HTRF assay signal was linear up to 80 min, and the 80-min assay signal was threefold higher than the background. The increased sensitivity of the HTRF assay allows not only a large savings in often precious enzymes and screening samples but also the titration of low picomolar inhibitors not measurable with the nanomolar quantities of enzyme required by SPA.

Effect of reagents on HTRF signal. While the SPA kinase assay measures signal directly from the radiolabeled peptide, the HTRF assay uses a secondary reagent, anti-phosphotyrosine antibody, to detect the phosphorylation of the peptide substrate. In other words, the magnitude of the HTRF signal reflects a combination of kinase activity and antibody affinity, while the SPA signal results exclusively from the kinase activity. Thus it is important for HTRF-based kinase assays to find the optimal substrate not only for the tyrosine kinase reaction but also for the antibody recognition. Several HS1 peptide analogs were synthesized based on the published peptide library results (21), and first tested in the SPA kinase assay to determine the relative activity on ZAP-70. Peptide A4 which contains YEEW instead of YEEV in the parent peptide (A1) showed a 25% increased activity with ZAP-70 (Table 1). Peptides A2 containing YWEW and A5 containing YFEW showed 3 and 12% decreased activity, respectively. The A3 peptide with YPEW showed ≈81% decreased activity. Since SPA does not use any secondary reagents (i.e., antibody, detection reagents), these numbers represent true relative activities for the ZAP-70 kinase reaction. In fact, the relative activities from the SPA were well matched with the ones from the gel electrophoresis assay which does not involve a streptavidin capture step (see above and Table 1). Therefore the streptavidin capture step in the SPA format does not alter the assay result. These peptides were then tested in the HTRF assay to determine the effect on the anti-phosphotyrosine antibody recognition step as well as the ZAP-70 kinase reaction. Surprisingly, peptide A5 increased the HTRF signal sevenfold. Since this peptide showed 12% decreased activity in the SPA, the sevenfold boost in the signal likely resulted from the increased antibody (PY20) affinity for this particular phosphorylated sequence. The signal observed in the HTRF assay is generated only from phosphorylated product that is also antibody bound. When the concentration of phosphorylated peptide is below the dissociation constant (K_d) for antibody recognition, the observed signal is proportional to the antibody affinity. Such affinities are specific for both the particular antibody and product sequence. To confirm that the difference in relative activity between SPA and HTRF assays resulted from the antibody affinity, K_d's for Eu(K)-PY20 and the phosphotyrosine peptides A1 and A5 were determined from a titration of the phosphopeptides with 0.25 nM of Eu(K)-PY20. Calculated K_d's of the phosphorylated A1 (pYEEV) and A5 (pYFEW) peptides were 5.5 and 0.9 nM, respectively. The difference in K_d's between the phosphopeptides was responsible for the sevenfold higher activity for peptide A5 in the HTRF assay. The increased HTRF activity for the A5 peptide was not observed when another anti-phosphotyrosine antibody, 4G10, was used (data not shown), implying that 4G10 shows no preference for the A5 sequence. Taken together, these results show that the HTRF format, in general, may require additional optimizations of secondary reagents (e.g., antibody) in comparison to the SPA format. The kinase reaction with 20 pM of ZAP-70 and the A5 peptide resulted in a greater than 15:1 signal-to-background ratio (Fig. 6). HTRF assay results for the other peptides can be explained in the same way.

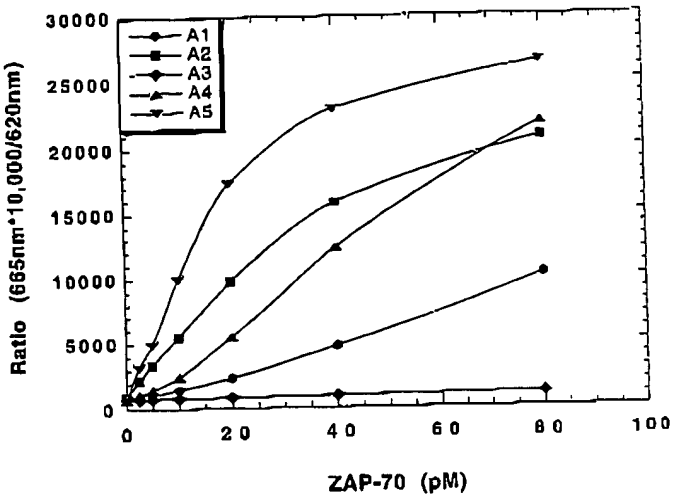

FIG. 6. Substrate peptide comparisons in HTRF ZAP-70 kinase assay. A parent HS1 peptide (A1) and it's analog peptides were tested in HTRF ZAP-70 kinase assay (see Table 1 for the detail sequence information). The kinase reaction was followed the general method described under Materials and Methods using 2.5–80 pM of ZAP-70. Ratio was calculated by (fluorescence at 665 nm × 10,000) / fluorescence at 620 nm.

Quench correction. Homogeneous assay techniques such as SPA and HTRF measure the signal directly from the assay well without any wash or separation steps. The measured signal can be interfered with by

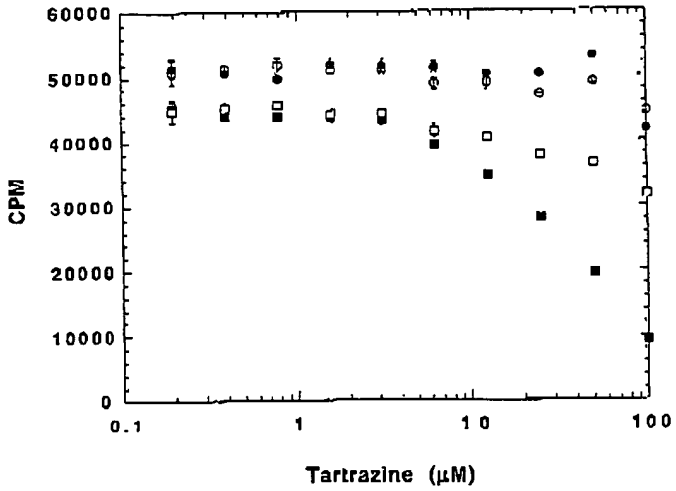

FIG. 7. SPA quench correction in centrifugation and bead floatation methods. Using the general kinase assay conditions described in the legend to Fig. 3, all the reactions were stopped by the addition of 50 μl of 20 mg/ml streptavidin-coated SPA beads in EDTA stop buffer. Either 100 μl CsCl (final concentration of 2 M) or 100 μl of EDTA stop buffer (without SPA beads) was added to the assay wells. The CsCl plate was incubated for 90 min at room temperature to float the SPA beads. The EDTA only assay plate was centrifuged for 5 min at 1000*g*. Both plates were counted using Packard's TopCount. ■, CPM, centrifugation method; ●, quench corrected CPM, centrifugation method; □, CPM, CsCl floatation method; ○, quench-corrected CPM, CsCl floatation method.

various colored samples in the assay wells, and as a result, the signal must be corrected for color quench. In the SPA assay, a general quench correction protocol (17) which was established by Packard and Amersham was used with some modifications. To derive an appropriate quench curve, identical SPA tyrosine kinase assay conditions were used with the exception that the yellow dye, tartrazine, was used instead of candidate kinase inhibitor samples. Although a variety of colored dyes could be used to generate a quench curve (17), tartrazine was chosen in this work because it has an absorption spectrum that overlaps the emission spectrum of the scintillant in PVT-SPA beads. The quench curve generated with tartrazine was stored in the TopCount to correct all the samples to quench-corrected CPM (QC-CPM). Figure 7 shows the color quenching effect by tartrazine up to 100 μM. Eighty and 30% of the signals were quenched by 100 μM dye in the centrifugation and CsCl floatation methods, respectively. After the quench correction was performed, the signals were effectively corrected for both the centrifugation method and the floatation method, showing that the use of CsCl has no effect on color quench correction. In the HTRF assay, such a color quench correction was not generally required since HTRF uses a ratio between two wavelengths (665 and 620 nm) which are measured simultaneously in the Discovery instrument. In theory, when the HTRF assay signal (665 nm) is absorbed by a colored sample, the internal standard Eu(K) signal (620 nm) is also absorbed to a similar degree. This assumes that color-producing absorption occurs over a broad wavelength range. While the absolute counts at each wavelengths decreased, the ratio should remain largely unaffected. To directly test this concept, several dye solutions which covered a broad wavelength range were tested in the HTRF kinase assay. Tartrazine (425 nm), methyl red (437 nm), methyl orange (543 nm), Chicago sky blue (618 nm), and naphthol green (714 nm) were each titrated in the stopped kinase assay mixture to prevent any possible inhibition of the kinase activity from the dye. The resulting HTRF ratio was only marginally affected by the colored samples even at concentrations up to 100 μM (data not shown). In fact, HTRF has been used to assay extremely colored samples such as natural products (22) and human sera (23).

Titration of inhibitors. A number of published as well as proprietary Merck inhibitors have been titrated in SPA and HTRF kinase assays to determine whether the IC_{50} values are similar for the two different assay formats. All the inhibitors showed similar IC_{50}'s between the two assay formats. Typical titration curves are shown in Fig. 8 for five src-family tyrosine kinases. Similar IC_{50}'s for the inhibitor A were found for both SPA and HTRF assay formats. The Hill coefficients were all close to 1 which was consistent with noncooperative inhibition of a single binding site in the src kinases. In addition, inhibition data of known tyrosine kinase inhibitors in both SPA and HTRF assays agreed well with the published data. For example, IC_{50}'s for the well-known tyrosine kinase inhibitor, staurosporine, are 9.3 and 10.5 nM for Lck from SPA and HTRF assay formats, respectively, and are very similar to published values (24).

Assay miniaturization. Assay miniaturization has become a very important factor in high-throughput screening. The move to smaller assay volumes reduces consumption of precious file compounds, enzymes, and other expensive reagents. In addition, total assay throughput can be increased by using higher-density assay plates in the miniaturized assay. As a step toward this goal of miniaturization, SPA and HTRF assays were performed in 384-well assay plates. The assay conditions were exactly the same as 96-well assay conditions except for decreased assay volumes. Total assay volumes were reduced from 200 μl in 96-well format to 50 μl for 384-well SPA or 32.5 μl for 384-well HTRF formats. In the 384-well SPA format, the assay signal was reduced fourfold as the assay volume was reduced fourfold. Accompanying this reduction, the background signal was also reduced fourfold. This resulted in a signal-to-background ratio of 7.3 which is

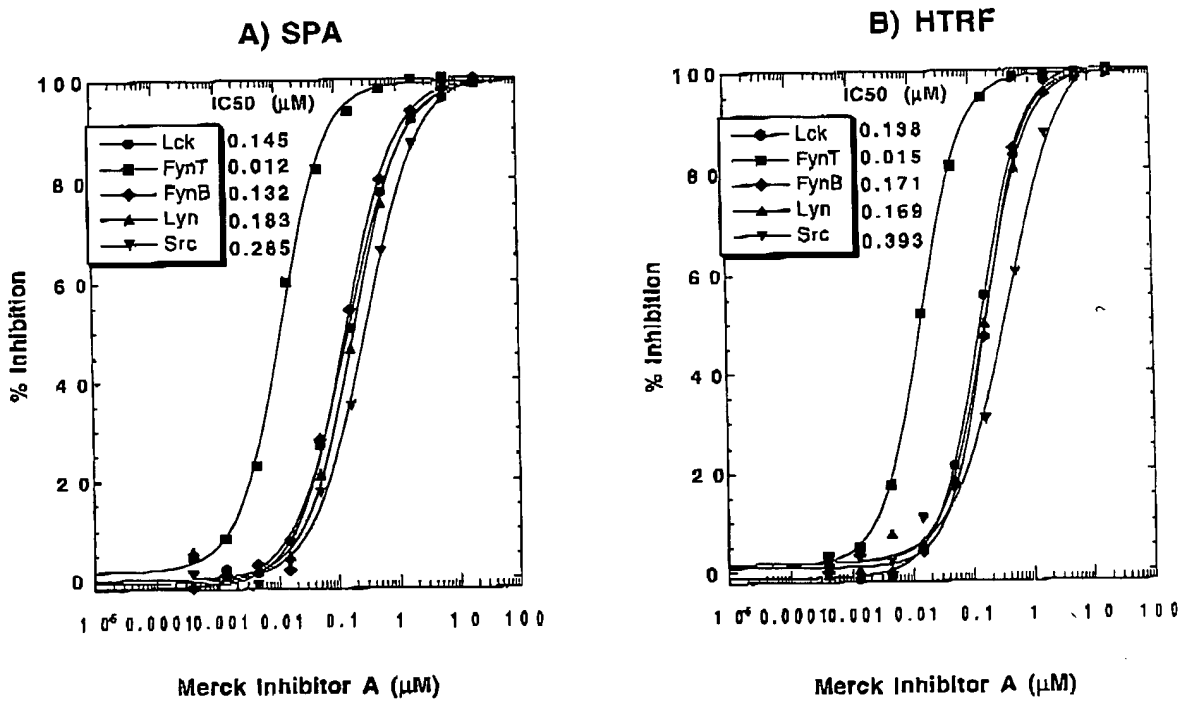

FIG. 8. Titration of a novel Merck kinase inhibitor A in SPA and HTRF assay formats. Inhibition of lck, fynT, fynB, lyn, and src activities were measured at 2 μM ATP, 0.75 μM peptide for SPA, and at 10 μM ATP, 0.75 μM peptide for HTRF.

very similar to that from the 96-well assay, 7.7. The 384-well HTRF assay also showed signal-to-background ratio comparable to that from the 96-well HTRF assay. The signal-to-background ratios are 7.0 for 384-well format and 9.5 for the 96-well format. Both SPA and HTRF 384-well assay formats have been semiautomated using a Tecan Genesis pipeting station, and the resulting data are very similar to the data from manual assay. Since the Discovery instrument uses a pencil-thin laser beam, the HTRF assay has high potential for even further miniaturization.

CONCLUSION

Two different homogeneous kinase assay formats, SPA and HTRF, were evaluated and compared. Both assay formats are proximity assays in which the assay signal is detected only when the energy donor (radioactivity for SPA, Eu for HTRF) and the energy acceptor molecules (SPA beads for SPA, allophycocyanin for HTRF) are in close proximity. The proximal nature of these assays allows them to be homogeneous, and thus do not require the removal (washing or filtration) of the excess free label. The homogeneous assays are, therefore, more robust and easier to automate than traditional assays (e.g., filter binding assay, ELISA assay). Several advantages and disadvantages were found in the SPA as well as the HTRF tyrosine kinase assays. Since SPA used fewer secondary reagents than HTRF (i.e., XL665, PY20-Eu), SPA required less labor for preparation of the secondary reagents and was easier to optimize than the HTRF assay. This requirement for the secondary reagents may cause an extension in the development time for an HTRF assay and may mean that not every assay will be adaptable to this format. For the same reason, the SPA format is likely to have fewer false positives from the inhibition of the secondary interactions. On the other hand, the HTRF format also has a number of advantages over SPA. Since HTRF uses no radioactivity as in SPA, it will be much safer to run the assay and cheaper to handle the liquid waste. Under the optimized tyrosine kinase assay conditions we obtained, the HTRF format resulted in two orders of magnitude more sensitivity than the SPA format. This allows a savings in precious enzymes and an ability to test subnanomolar inhibitors which was not possible in SPA format. The Discovery instrument (Packard) requires only 1.5 min to read a 96-well plate in HTRF, while TopCount (Packard) needs 20–40 min. The fast reading time allows a reduction in the assay optimization time and removes one of the bottleneck steps in high-throughput screening. The assay miniaturization to 384-well format was successful for both SPA and HTRF tyrosine kinase assays. Both resulted in similar signal-to-background ratios compared to the

96-well format. Both assays have been automated for faster screen execution. In conclusion, both SPA and HTRF can improve the throughput and the accuracy of screening for drug leads. The availability of appropriately labeled reagents will largely determine which method is chosen.

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